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Nasal delivery of antigen with the B-subunit of *Escherichia coli* heat-labile enterotoxin augments antigen-specific T cell clonal expansion and differentiation.

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Abbreviations: Etx, *Escherichia coli* heat-labile enterotoxin; Ctx, Cholera toxin; EtxB, *Escherichia coli* heat-labile enterotoxin B-subunit; CtxB, Cholera toxin B-subunit; CLN, cervical lymph node; i.n., intranasally; ADP, adenosine diphosphate; NALT, nasal associated lymphoid tissue

Summary

The ability of the B-subunit of *Escherichia coli* heat-labile enterotoxin (EtxB) to act as an adjuvant for antigens co-administered intranasally (i.n.) was investigated using the DO11.10 adoptive transfer model where OVA-specific T cells can be monitored directly. Intranasal delivery of OVA with EtxB to these mice caused increased T cell proliferative and systemic antibody responses against antigen. The increased Th2-cytokines detected following *in vitro* restimulation of splenocyte and cervical lymph node (CLN) cells from immunised mice correlated with increased IgG1 OVA-specific antibody production. Flow cytometric analysis of the T cells from mice early after immunisation directly revealed the ability of EtxB to support antigen-specific clonal expansion and differentiation. Furthermore, while responses were first detected in the CLN, they rapidly progressed to the spleen where they were further sustained. Examination of CD69 expression on the dividing cells supported the notion that activation induced by the presence of the antigen is not sufficient to drive T cell differentiation. The lack of CD25 expression on dividing cells suggests that EtxB-mediated T cell clonal expansion may occur without a sustained requirement for IL-2.

1 Introduction

Mucosal delivery of most soluble proteins triggers specific tolerance rather than immunity. This fact necessitates the identification of suitable adjuvants for mucosal vaccines. Both *Escherichia coli* heat-labile enterotoxin (Etx) and its relative cholera toxin (Ctx) act as potent mucosal adjuvants potentiating local and systemic immunity to admixed antigens [1, 2]. Etx and Ctx consist of a single A subunit and five identical B subunits. In the resulting hexamers the A subunit is associated with a doughnut-shaped ring formed by the five identical B subunits. The B-subunit pentamer is an extremely stable non-covalently associated protein complex [3]. While the A subunit has adenosine diphosphate (ADP)-ribosyl transferase activity and is responsible for toxicity, the B subunits are non-toxic. Their primary function lies in binding receptors, particularly GM1 ganglioside, facilitating internalisation of the A subunit into the cell.

The use of the native toxins as adjuvants in humans is clearly not possible due to their inherent A-subunit mediated toxicity. Attempts to take advantage of their adjuvant properties while avoiding toxicity have therefore focused either on the use of the recombinant B subunits alone [4, 5] or on mutants with reduced or absent ADP-ribosyl transferase activity [reviewed in 6]. Although initial data derived by these approaches were discouraging [8] more recent studies have supported the potency of both mutant molecules [9, 10] and the B-subunit of Etx (EtxB) [4, 5] as adjuvants. EtxB augmented both systemic and mucosal antibody responses following i.n. delivery with either HSV-1 glycoproteins [4] or hen egg lysozyme [5]. *In vitro* restimulation of lymph node cells from mice immunised with EtxB as adjuvant resulted in increased proliferation and cytokine production [4]. Further, i.n. immunisation using EtxB as an adjuvant was extremely effective at reducing protecting against ocular infection with HSV-1. Interestingly, cholera toxin B-subunit (CtxB) failed to augment T cell or antibody responses to either HSV-1 glycoproteins or HEL at a wide range of doses [4, 5].

The processes by which EtxB modulates the immune response to co-administered antigen are unclear. Receptor-binding by the B-subunit plays a critical role, as demonstrated by the failure of a non-receptor binding mutant of EtxB, EtxB(G33D) [10], to potentiate a response to HEL [5]. It is therefore widely assumed that receptor interactions with mucosal epithelial cells trigger translocation into the underlying lymphoid tissues where modulation of leukocytes affects the activation and differentiation of antigen-responsive T cells. Determination of the events involved

requires the use of a model in which the early processes of specific T cell activation can be monitored *in vivo*.

An adoptive transfer system where ovalbumin $(OVA)_{323-339}$-specific T cells from the T cell receptor transgenic DO11.10 mouse [11] are injected into normal BALB/c mice has been used to monitor the fate of antigen specific T cells *in vivo* [12]. Studies using this system have investigated the processes associated with both the generation of an active immune response and the induction of tolerance [13-15]. This model has the advantage of allowing the identification of antigen-specific cells in an animal in which normal homeostatic controls are present, and in which there is a diverse repertoire of potential antigen-specificities within both the T cell and B cell compartments. We have therefore employed this system to monitor T cell activation, differentiation and clonal expansion following nasal administration of OVA in the presence or absence of EtxB. We demonstrate that EtxB possesses the ability to drive enhanced T cell division and differentiation.

2 Results

2.1 The ability of EtxB to act as an adjuvant for OVA

Experiments were performed to confirm that EtxB acts as an adjuvant for OVA in DO11.10 chimeric BALB/c mice. Twenty-four hours after adoptive transfer, $CD4^+KJ1\text{-}26^+$ cells represented approximately 1% of the $CD4^+$ T cells in the spleen and lymph nodes (mesenteric, cervical and inguinal) of recipient mice (data not shown). Recipients were given three i.n. immunisations at one-week intervals with either EtxB, OVA or PBS alone, or OVA in the presence of EtxB or EtxB(G33D). 15 days after the final immunisation, T cell proliferative and cytokine responses were monitored in cultures of both splenic and CLN cells. While proliferation above background was detected in cultures from all mouse groups (Fig. 1A) a strong reaction indicative of a recall response was only observed in those that had received OVA with EtxB. The addition of EtxB to OVA also markedly enhanced cytokine responses. While splenic cultures from all groups did produce some cytokine above background (most notably IFNγ), high levels of IL-4 and IL-10 in addition to IFNγ were only seen in cultures from OVA+EtxB immunised mice (Fig. 1B). Similarly, IL-4 and IL-10 were detected at high levels in cultures of CLN cells from OVA+EtxB treated mice, but not other groups. IFNγ was detectable again in CLN cultures only from the OVA+EtxB treated mice. Similar results were obtained in four separate experiments.

Comparison of OVA-specific IgG1 and IgG2a levels in serum taken from the different immunisation groups, revealed insignificant levels in mice that were not immunised with OVA (Fig. 2). Exposure to OVA, even in the absence of EtxB, or with EtxB(G33D), significantly increased the levels of IgG1 ($p<0.05$ versus the PBS or EtxB group) but not IgG2a. The addition of EtxB to OVA caused a much stronger IgG1 response ($p<0.05$ versus OVA and $p<0.01$ versus OVA+EtxB(G33D)). IgG2a antibodies were not increased in any group.

2.2 Analysis of cell surface markers following intranasal immunisation

Having established that EtxB acts as a potent mucosal adjuvant in DO11.10 chimeras, experiments investigated its effects on T cell activation and differentiation. DO11.10 chimeras were immunised as described above on days 1 and 8. The expression of CD69, CD25 and CD45RB on $CD4^+KJ1\text{-}26^+$ cells was monitored in the spleen, mesenteric, cervical and inguinal lymph nodes 1 and 5 days later. Data from 3 separate experiments

on 3 separate mice per group were analysed similarly and have been pooled to produce Fig. 3.

CD69 is an acute activation marker. When mice were given PBS alone a smaller number of $CD4^+KJ1\text{-}26^+$ cells expressed CD69 in the spleen (8%) compared to the CLN (25%). Administration of OVA either with or without EtxB or EtxB(G33D) caused a marked increase in the proportion of $CD4^+KJ1\text{-}26^+$cells co-expressing CD69 in the CLN on days 1 ($p<0.05$ versus PBS) and 5 ($p<0.001$ versus PBS). EtxB alone did not increase CD69 expression ($p>0.05$ versus PBS). Furthermore, co-administration of EtxB with OVA did not result in any further increase to that observed with OVA alone. No alterations in CD69 expression were observed in the spleen.

The IL-2Rα chain, CD25, is expressed on activated T cells and endogenous T regulatory cells [16]. A similar small percentage of $CD4^+KJ1\text{-}26^+$ T cells expressing CD25 were detected in the spleen and CLN of all groups of mice (Fig. 3).

Levels of CD45RB decrease as T cells differentiate following activation [17]. The percentage of cells with low levels of CD45RB expression among the $CD4^+KJ1\text{-}26^+$ population was determined. On day 1 the percentage of $CD45RB^{low}CD4^+KJ1\text{-}26^+$cells increased in the CLN of mice that had received OVA (Fig. 3). The greatest increase was observed in the OVA+EtxB group ($p<0.05$ compared to PBS or EtxB treated mice). Similar changes observed in the spleen on day 1 were not statistically significant. On day 5, mice given OVA+EtxB had significantly increased $CD45RB^{low}$ cells in both the spleen and the CLN compared to mice that were given OVA alone or with EtxB(G33D) ($p<0.05$ and $p<0.05$ versus OVA and $p<0.001$ and $p<0.05$ versus OVA+EtxB(G33D) for spleen and CLN respectively). The percentage of $CD45RB^{low}$ cells in the mice that were given OVA in the absence of EtxB was also increased when compared with the control PBS group, although these differences were not significant. It should be noted that no changes were observed to the phenotypes of the $CD4^+KJ1\text{-}26^-$ T cells in these experiments, and analysis of cells isolated from the inguinal and mesenteric lymph nodes failed to reveal any differences (data not shown).

2.3 **EtxB increases the responder frequency in the CLN and spleen**

The vital dye CFSE was used to determine if EtxB altered clonal expansion in response to OVA [18]. DO11.10 T cells were CFSE-labelled prior to i.v. transfer into normal BALB/c mice, which were then immunised as previously. At days 1 and 5 following the

second immunisation, spleen and CLN cells analysed by flow cytometry. No divisions occurred in the groups that received PBS or EtxB alone (Fig. 4A). In the remaining three groups divisions were observed in the CLN but not in the spleen on day 1 and in both tissues on day 5. For the CLN a very low number of cells were in the first cycles of division on day 1 in all the three groups. Administration of EtxB with OVA did not appear to enhance cell division in the CLN at that time point. On day 5 more division had occurred in the CLN. Co-administration of EtxB increased cell division in the CLN on day 5. The results were similar for the spleen at that time point (day 5). While, divisions in the spleen were evident in all three groups, increased numbers of cells at late stages of divisions were observed in the OVA+EtxB immunised mice. In all three groups divisions in the spleen had progressed to later rounds compared to those in the CLN.

The CFSE profiles from two separate experiments each including 3 mice per group were used to calculate the responder frequency and the proliferative capacity of the $CD4^+KJ1\text{-}26^+$ population (Fig. 4B). The analysis of the responder frequency showed a higher proportion of transferred cells divided in the spleen and CLN from the OVA+EtxB group than from the other two groups. This was most evident in the spleen ($p<0.01$). Examination of the proliferative capacity showed that there were no differences between the numbers of daughter cells generated per precursor between the three groups for each tissue. The proliferative capacity was higher in the spleen in all groups.

2.4 Expression of cell surface molecules on dividing cells

Co-staining experiments were carried out to investigate the association between altered phenotype and cell division. When CD69 expression was examined simultaneously with CFSE fluorescence, the results showed that, in all groups, upregulation of CD69 preceded entry into cell division in the CLN (Fig. 5A). As cell divisions continued, CD69 expression was progressively reduced. Divisions in the spleen were not accompanied by the expression of CD69 on the cell surface. Very few splenic $CD4^+KJ1\text{-}26^+$ cells expressed CD69, and those that did were undivided cells of which similar numbers were seen in the PBS control group as were seen in groups receiving OVA. CD25 expression was not associated with the phenotype of dividing cells either in the spleen or in the CLN. A small number of $CD25^+CD4^+KJ1\text{-}26^+$ could be detected

in all mouse groups, and the majority of dividing cells did not express CD25 (data not shown).

Analysis of CD45RB expression showed that, divisions in both the spleen and the CLN were accompanied with a sequential reduction in CD45RB expression while CD45RB levels remained high in the groups where no divisions were observed (PBS; Fig. 5B and EtxB; data not shown). The simultaneous reduction in CFSE fluorescence and CD45RB expression during division was particularly evident in the mice that were given OVA+EtxB where both the numbers of divisions were increased and more cells exhibited a differentiated phenotype ($CD45RB^{low}$).

2.5 Correlation between increased T cell proliferation and CD45RB expression

The increased ability of cells from OVA+EtxB treated mice to proliferate *in vitro* in response to OVA could be the result of clonal expansion leading to an increased precursor frequency in the tissues of these mice and/or the enhanced differentiation state of the cells allowing a more vigorous response. In order to investigate this, staining for CD45RB expression among the $CD4^{+}KJ1\text{-}26^{+}$ T cells was performed at the same time point that the tissues were used for *in vitro* stimulation with OVA. When the overall percentages of $CD4^{+}KJ1\text{-}26^{+}$ among the CD4 population were compared between the various groups the numbers were highest in both the spleen and the CLN of the OVA+EtxB immunised mice. However, the differences between the groups were small (Fig. 6). OVA immunisation led to an increase in the proportion of the $CD4^{+}KJ1\text{-}26^{+}$ that were $CD45RB^{low}$, with the highest percentage being observed in the group that had received OVA+EtxB.

3 Discussion

The ability of EtxB to enhance the immune response to intranasally co-administered antigens is demonstrated here in DO11.10 chimeras. The addition of EtxB to OVA significantly increased both antibody production and T cell proliferative and cytokine responses against OVA. The induction of high levels of IgG1 anti-OVA antibodies in the absence of detectable IgG2a indicates a Th2-dominated response when EtxB is used as adjuvant. This was reflected by stimulation of IL-4 and IL-10 production by splenic and CLN cells, and is consistent with data from the use of EtxB as an adjuvant for HSV-1 glycoproteins [4]. As with HSV-1 antigen, the Th2-dominated antibody response occurred despite the presence of lymph node IFNγ production, an observation indicating that the balance between Th1 and Th2 cytokines, rather than their presence or absence, determines the nature of the antibody response. Interestingly, the level of IFNγ produced in spleen cell cultures was relatively high in all groups, with similarly enhanced levels being observed in all those that had received OVA. It is likely that this reflects the capacity of naïve T cells to produce IFNγ in primary responses that would occur in all of these cultures, and that exposure to OVA *in vivo* led to partial priming of the splenic cell population for IFNγ production regardless of the presence of EtxB. Importantly, the adjuvant activity of EtxB was entirely dependent on receptor-binding, as EtxB(G33D) [10] failed to enhance both antibody and T cell mediated responses against OVA.

Analysis of OVA-specific T cells revealed that EtxB markedly altered T cell activation, differentiation and clonal expansion in response to nasally administered antigen. In contrast to studies of other adjuvants and immunisation routes using the same model, there was not, however, a significant increase in the overall numbers of KJ1-26 positive cells in either the spleen or local lymph node (data not shown). Marked increases in OVA-specific cell numbers have been reported following intravenous administration of OVA peptide, subcutaneous injection and oral feeding of OVA [12, 19, 15]. In these reports, the presence of the antigen was adequate to induce significant clonal expansion, which was further increased when adjuvants such as LPS [20, 21], CFA [21] or Ctx [15] were added. The lack of such a difference in our system may reflect the route of administration, the timing of our observations, which followed a second antigen dose, or more probably the much lower dose of antigen that was used. The dose of OVA used by us induced a functionally significant response in the presence

of EtxB as displayed by the antibody and proliferation data, and was in keeping with the quantities of antigen used in other studies with this adjuvant [4, 5]. Therefore, the observations that we have made are physiologically relevant to the induction of the types of responses that we sought to study.

Phenotypic analysis of antigen specific T cells in the spleen and CLN following OVA administration showed that cell activation was associated with some upregulation of CD69 expression, but no increase in the proportion of OVA-specific cells expressing CD25. Co-staining in studies with CFSE confirmed that dividing KJ1-26$^+$ cells did not express CD25. IL-2 usually drives the proliferative phase of the T cell response [22] and, as divisions progress the requirement for IL-2 diminishes and cells down-regulate CD25 [23]. Although our observations were unexpected, data describing T cell proliferation in IL-2 deficient mice with LPS as adjuvant [24] correlate with this finding. Indeed, IL-2 not only acts as a T cell growth factor, but is also a key factor in priming cells for activation-induced cell death [25], a process that prevents the excessive accumulation of activated T cells. Accordingly, IL-2 deficient mice show evidence of dysregulated immune responses and autoimmunity. Avoidance of this pathway by the failure of T cells, activated as a result of the co-administration of EtxB, to upregulate CD25 may promote more persistent responses.

As expected, cell division was detectable in the CLN but not the spleen one day after antigen administration. T cells initiating divisions in the CLN had upregulated CD69 expression irrespective of the administration of EtxB. This is consistent with reports that transient activation occurs during the induction of tolerance [15, 16, 18, 19]. Despite the administration of OVA, either alone or in the presence of EtxB(G33D), being sufficient to stimulate CD69 upregulation, receptor-binding by EtxB was required to stimulate optimal differentiation and division. Thus, down-regulation of CD45RB was much more marked in animals given OVA with EtxB, and the proportion of cells with lowered levels of CFSE was much higher in this group. On day 5, large numbers of cells were also dividing in the spleen of the OVA with EtxB mice, although these were not expressing CD69. Indeed CD69 expression was not observed on undivided or dividing KJ1-26$^+$ spleen cells in any of the groups.

Quantitation of CFSE profiles revealed that EtxB increased the responder frequency rather than the proliferative capacity of OVA-specific T cells in the spleen and the CLN. The increased responder frequency was more evident in the spleen where very few divided cells were detected in mice that did not receive OVA with EtxB.

Interestingly, the addition of EtxB gave rise to essentially the same precursor frequencies in both spleen and CLN. Moreover, the proliferative capacity was consistently lower in the CLN compared to the spleen for each group that received OVA. While this may indicate that cells entering cell cycle in the spleen are more likely to go through multiple divisions than those in the CLN, it should be noted that the analysis does not take into account cell migration. Thus, the data may also indicate that cells migrate to the spleen following activation and initial cell division in the CLN. The latter possibility is also suggested by the lack of dividing spleen cells on day 1 and the absence of upregulated CD69 expression in this tissue.

The rapid regulation of CD69 expression means that its presence probably indicates presentation of antigen locally. The absence of CD69 expression in the spleen strongly suggests that, at this dose, local presentation of OVA is not the primary mechanism initiating cell division in this tissue. Further, the data suggest that CD69 expression is lost from activated cells as they migrate to the spleen from peripheral lymph nodes. Taken together with the fact that we observed stronger proliferative responses to OVA in the spleen, the findings suggest that this tissue is a major site of homing of activated cells after such immunisation. However, it is also likely that some component of the activated T cell population re-enters the mucosal tissues associated with the nasal cavity in order to support the local production of secretory antibody that is seen there [4, 5]. Studies of the NALT (nasal associated lymphoid tissue) will be necessary to confirm this, and an investigation of chemokine receptor expression by differentiating KJ1-26$^+$ cells would be interesting.

How does EtxB promote T cell differentiation and division? Mechanisms associated with adjuvant function include the production of inflammatory cytokines [24, 42], the stimulation of APC to express co-stimulatory molecules [25] and the control of dendritic cell migration [44]. EtxB has multiple activities on cells of the immune system as well as on mucosal epithelial cells. These include the ability to trigger polyclonal upregulation of class II MHC and CD86 on B cells and to modulate APC cytokine production. In addition, Ctx has been shown to be capable of contributing directly to T cell activation through negating the requirement for costimulation [48]. The quantities of free EtxB entering the lymph nodes following intranasal delivery are likely to be very low and therefore the key effects of EtxB are probably associated with modulation of APC. The capacity to activate B cells may enhance their involvement in expanding the activated T cell response, and help shape their differentiation toward Th2 cells. It is

conceivable that this effect alone may be sufficient to allow transiently activated T cells, which are initially stimulated in a manner identical to that which occurs after mucosal antigen delivery alone, to undergo productive differentiation rather than becoming abortively activated. Alternatively, EtxB may also modify the activity of dendritic cells. While this has yet to be tested, whole Ctx is known to modulate dendritic cell function favouring Th2 differentiation [49] and EtxB can trigger TNFα and IL-10 production by monocytes while at the same time inhibiting IL-12 secretion [51]. Such a profile would be consistent with activating T cells but failing to promote Th1 differentiation. Elucidation of the precise effects of EtxB on APC populations *in vivo* will be necessary to determine what receptor-mediated activities are the key to its capacity to promote T cell activation, differentiation and cell division. Such an understanding will allow the rational use of EtxB as a potentially non-toxic adjuvant for the development of important mucosal vaccines.

4 Materials and Methods

4.1 Experimental animals

Female BALB/c mice (Harlan Olac, Bicester, U.K.) and DO11.10 TCR-transgenic mice (bred at the University of Bristol animal facilities) were used at between 8 and 10 weeks of age.

4.2 Reagents

Recombinant EtxB and EtxB(G33D) were purified and depleted of LPS as reported previously [51]. The LPS content was routinely <30 EU/mg of protein. OVA (grade IV: Sigma) was reconstituted in PBS and dialysed extensively prior to use.

4.3 Adoptive transfers and immunisations.

$6x10^6$ T-lymphocytes (>85% $CD3^+$) isolated from the spleen and mesenteric lymph nodes of DO11.10 mice using nylon wool (Du Pont Biotechnology, Boston, U.S.A.) were i.v. into BALB/c mice. For some experiments, enriched T cells were labelled with CFSE (Molecular Probes, U.S.A) prior to adoptive transfer. On day 1 following adoptive transfer recipient mice were immunised i.n. with 100µg OVA with or without 20µg EtxB or EtxB(G33D), or with the B-subunits or PBS alone. Immunisations were repeated either on day 8 and 15, or on day 8 alone as indicated.

4.4 *In vitro* proliferation and cytokine assays

Spleen and CLN cells were prepared as described [52] and cultured in $25cm^2$ flasks in α-MEM (Gibco-BRL) supplemented with 20mM HEPES buffer, 100µg/ml streptomycin sulphate, 100U/ml benzyl penicillin, 4mM L-glutamine, 50 µM 2-mercaptoethanol (Sigma) and 0.5% autologous mouse serum with or without 200µg/ml OVA. At time points, triplicate 100µl samples were transferred to 96-well round bottom plates and pulsed with 18.5kBq/well of ^{3}H-thymidine (TdR) for 6 hours. Cells were harvested and ^{3}H-TdR incorporation was measured by standard scintillation and results are presented as mean cpm ± SEM. Production of IFNγ, IL-4 or IL-10 was detected by CelELISA, as previously described [53]. Cytokine production was estimated by linear regression calculated from a standard curve obtained with recombinant cytokine using Microplate Manager 4.0 (Bio-Rad Laboratories Inc.) software.

4.5 Measurement of OVA- specific antibody responses

Serum was obtained 15 days following a third i.n. immunisation. OVA coated plates were washed and blocked with 1% BSA/PBS before serial dilutions of serum were added. Subsequently, a horseradish peroxidase-conjugated antibody specific for IgG1 or IgG2a (Serotec) was added to the wells. Plates were developed with o-phenylenediamine dihydrochloride substrate (Sigma) and the optical densities of plate wells measured at 490nm. Amounts of anti-OVA immunoglobulin were estimated by endpoint titre, calculated from linear regression analysis of $\log_{10}$-transformed data using Statistics, Version W1.58 (Blackwell Scientific Publications, U.K.).

4.6 Flow cytometric analysis

Cells were stained with the following antibodies: CD45RB (16A; PharMingen), CD25 (PC61; PharMingen), CD69 (H1.2F3; PharMingen) and CD4 (CT-CD4; Caltag Labolatories) or a biotinylated anti-clonotypic antibody KJ1-26 [54]. ExtrAvidin-FITC or -PE (Sigma) or Streptavidin-APC (Caltag Laboratories) were used as necessary. Staining was performed in HBSS containing 0.02% sodium azide (Sigma) and 5% normal rat serum (Sigma). All analyses were performed with appropriate control samples including isotype/conjugate-matched controls. At least 2000 gated $CD4^+KJ1\text{-}26^+$ events were collected for each sample. Analysis of collected data files were made using WinMDI 2.8 (Dr. J. Trotter, Scripps Research Institute, U.S.A).

4.7 Quantitative assessment of T cell clonal expansion from CFSE profiles

Quantification of differences observed from CFSE profiles was achieved using a previously described mathematical model [55,56]. This model is based on the principle that the size of the antigen-specific daughter T cell subset and the size of the precursor subset are related by a function of 2^n, where n is the number of division cycles achieved during clonal expansion. The events under each CFSE fluorescence peak represent a proportion of the Ag-specific T cell population that have undergone n division cycles. If A is the total number of clonally expanded T cells at the peak of the response then the percentages of cells under each fluorescence peak can be used to calculate the absolute number of T cells under each peak (En) as a percentage of A. The absolute number of precursors that gave rise to that number of cells can then be calculated by dividing the absolute number of T cells with 2^n where n is the number of divisions corresponding to that peak. The sum of the precursors for each division cycle or Ps can be calculated

from the above (Equation 1), which gives the size of the precursor sample pool that have generated the cell sample with the given division pattern. Similarly, the size of the precursor sample pool that has responded by dividing (Psr) can be determined by summing the absolute numbers of precursors from 1 to n division cycles (Equation 2).

$$Ps = \sum_{0}^{n}\left(\frac{En}{2^n}\right) \quad (1)$$

$$Psr = \sum_{1}^{n}\left(\frac{En}{2^n}\right) \quad (2)$$

The parameters of clonal expansion that can be calculated with the use of the above are the responder frequency (R) (Equation 3) and the proliferative capacity (Cp) (Equation 4) representing the proportion of the precursor sample pool that responded to antigenic stimulation by dividing and the number of cells generated by the average responder cell respectively:

$$R = \frac{Psr}{Ps} \quad (3)$$

$$Cp = \frac{\sum_{1}^{n} En}{Psr} \quad (4)$$

As both the frequency and the proliferative capacity are independent of the net yield of Ag-specific clonally expanded T cells measured at the peak of the response and described as *A* for reasons of simplicity calculations did not include the arithmetic value of A although these numbers were measured during the experiments.

4.8 Statistical analysis

Descriptive statistics and normality testing were calculated using Graphpad Prism 3.02 (Graphpad Software Co., U.S.A.). For the comparison of multiple groups of parametric data one-way analysis of variance (ANOVA) was used. If $P<0.05$ for ANOVA, the Newman-Keuls for multiple comparison post-test was used to determine statistical significance for each treatment group in comparison to the others. For all statistical analysis, a P value of less than 0.05 was considered significant.

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Figure legends

Fig. 1. (A) Intranasal exposure to OVA in the presence of EtxB enhances proliferation following stimulation with OVA *in vitro*. Isolated T cells from DO11.10 mice were adoptively transferred into normal BALB/c mice (day 0). On days 1, 8 and 15, the recipients were immunised intranasally with OVA (open diamond; O), OVA+EtxB (open triangle; O+E) or OVA+ EtxB(G33D) (open square; O+G) (day 1). Control mice were given PBS (closed diamond; P) or EtxB (closed circle; E). On day 30, splenic and CLN cells cultured *in vitro* with OVA. Proliferation was measured and the data are expressed as mean values ± SEM from triplicate samples. Maximal proliferation in cultures established in the absence of OVA is indicated by a dotted horizontal line. The results shown are representative of four identical experiments including 5 mice per group each. (B) Cytokine production at the peak day of the response, day 5 for the spleen and day 7 for the CLN. Data are expressed as mean values ± SEM from triplicate samples. Maximal levels of the respective cytokines detected in cultures established in the absence of OVA are indicated by a dotted horizontal line.

Fig. 2. Exposure to OVA in the presence of EtxB enhances antibody production. Following the adoptive transfer and immunisation protocol used for the *in vitro* cultures (Fig. 1) mice were bled 15 days following the third immunisation and the levels of OVA-specific antibody were quantified by isotype-specific ELISA. Endpoint titers were calculated by linear regression and the data from 20 mice in each group (5 mice in each of 4 identical experiments) were combined. The median titre per group is indicated by a horizontal bar.

Fig. 3. Phenotype of $CD4^+KJ1\text{-}26^+$ T cells following stimulation *in vivo* with OVA and EtxB. Recipients of DO11.10 T cells were immunised intranasally on days 1 and 8 with OVA alone (O) or with OVA+EtxB (O+E), OVA+EtxB(G33D) (O+G). Control mice were given PBS (P) or EtxB (E). Flow cytometric analysis of the spleen and CLN was performed 1 and 5 days and the expression of CD69, CD45RB and CD25 on $CD4^+KJ1\text{-}26^+$ cells from individual mice was analysed. The data represent the mean of 9 individual mice (3 separate experiments including 3 mice per group each) ± SEM.

Fig. 4. (A) Division of OVA-specific $CD4^+$ T cells following immunisation. DO11.10 T cells were labelled with CFSE and transferred into BALB/c mice. Recipients were immunised as described in Fig 3. At days 1 and 5 after the second immunisation, cell division by $CD4^+$ KJ1-26^+ cells was assessed. The data shown correspond to individual mice from each group and are representative of two separate experiments with 3 mice per group. (B) Responder frequency and proliferative capacity of adoptively transferred cells following immunisation. The CFSE profiles derived from the spleen and CLN of mice immunised with OVA alone or with EtxB/EtxB(G33D) were used to deduce the responder frequency and proliferative capacity. Data from 6 individual mice from each group in two separate experiments are presented as mean ± SEM.

Fig. 5. (A) Association of cell division and CD69 expression in the OVA-specific $CD4^+$ T cell subset following stimulation in vivo with nominal antigen. CFSE-labelled DO11.10 T cells were transferred into BALB/c mice. Recipients were immunised as described (Fig. 3). The expression of CD69 and the division of CFSE fluorescence were estimated simultaneously on gated $CD4^+$KJ1-16^+ T cells at the indicated time points and tissues. The data shown are from an individual mouse from each group but are representative of 6 mice investigated in two separate experiments. (B) Association of cell division and CD45RB expression in the OVA-specific $CD4^+$ T cell subset following stimulation *in vivo* with antigen.

Fig. 6. Persistence and state of differentiation of $CD4^+$KJ1-26^+ cells. Adoptive transfer, immunisations, and flow cytometric staining were as already described (Fig. 1 and 3). 15 days following the third immunisation samples from the spleen and CLN of the immunised mice were stained with anti-CD4, anti-KJ1-26 and anti-CD45RB antibodies. The percentages of $CD4^+$KJ1-26^+ cells and the percentage of $CD45RB^{low}$ cells among them were determined as previously in density plots. The data are expressed as mean values ± SEM. from 5 mice for the spleen, while for the CLN values were derived from mixed samples of the 5 mice. The results shown are representative of two experiments.

Figure 1

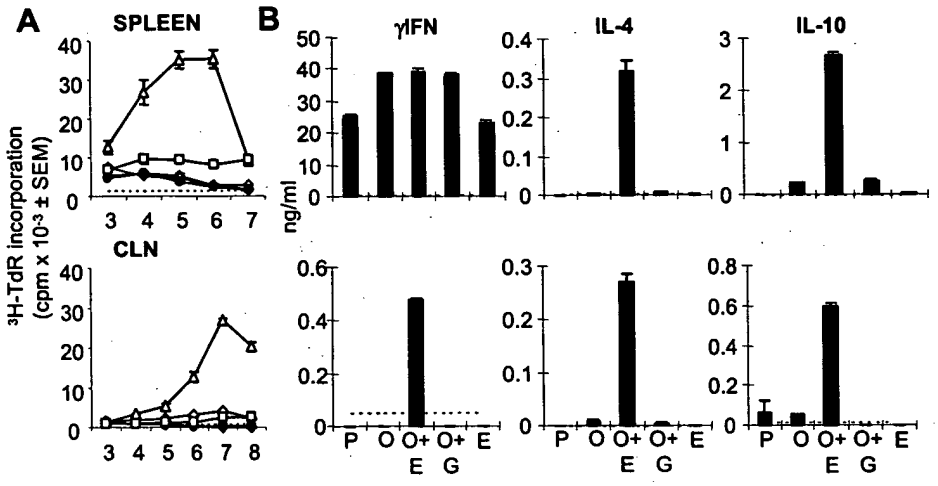

A
SPLEEN
CLN
^{3}H-TdR incorporation (cpm x 10^{-3} ± SEM)
B
γIFN
IL-4
IL-10
ng/ml
P O O+ E O+ G E

Figure 2

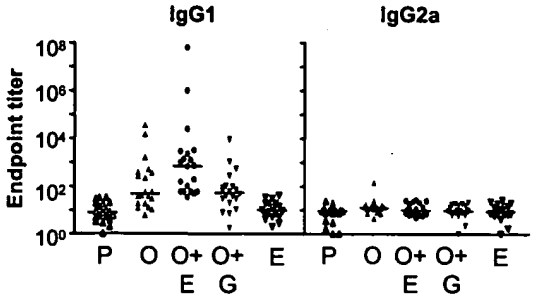

IgG1
IgG2a
Endpoint titer
10^8
10^6
10^4
10^2
10^0
P
O
O+ E
O+ G
E
P
O
O+ E
O+ G
E

Figure 3

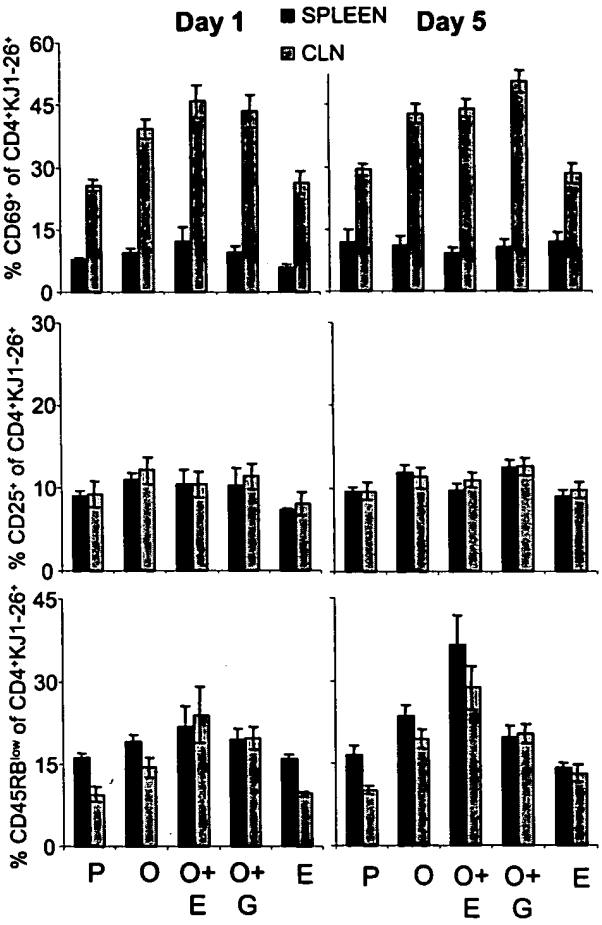

Day 1
SPLEEN
CLN
Day 5
% CD69+ of CD4+KJ1-26+
% CD25+ of CD4+KJ1-26+
% CD45RBlow of CD4+KJ1-26+
0
15
30
45
60
10
20
P
O
O+ E
O+ G
E

Figure 4

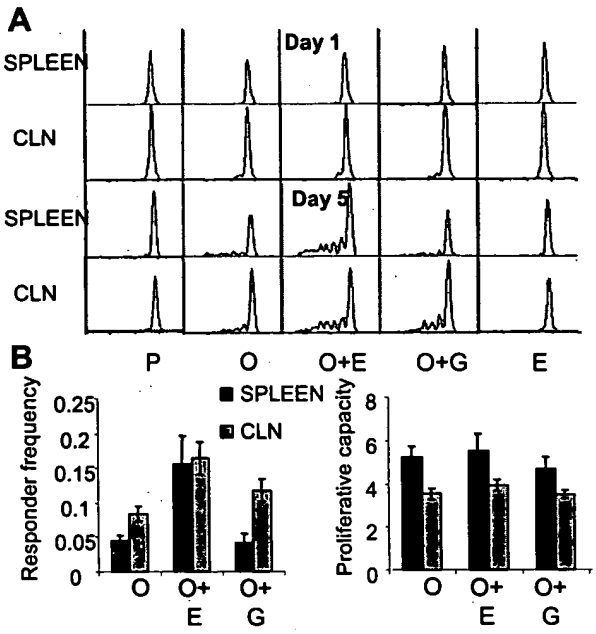

A
Day 1
SPLEEN
CLN
Day 5
SPLEEN
CLN
P
O
O+E
O+G
E
B
SPLEEN
CLN
Responder frequency
0.25
0.2
0.15
0.1
0.05
0
O
O+ E
O+ G
Proliferative capacity
8
6
4
2
0
O
O+ E
O+ G

Figure 5

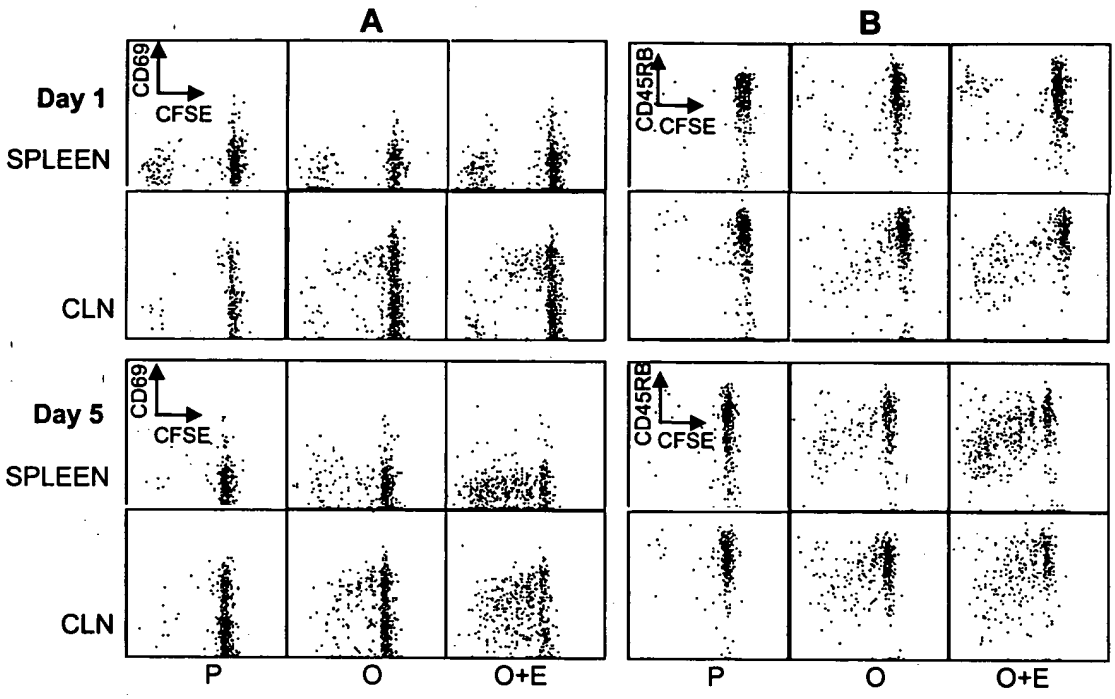
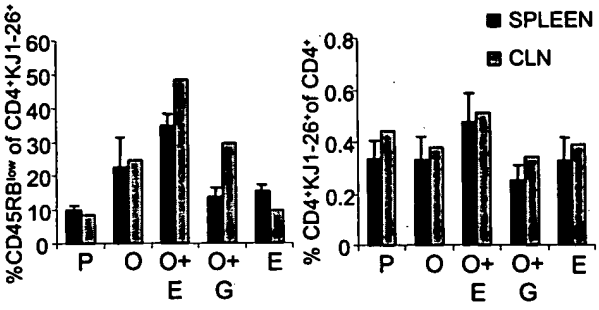

A
B
Day 1
SPLEEN
CLN
Day 5
SPLEEN
CLN
CD69
CFSE
CD45RB
CFSE
P
O
O+E
P
O
O+E

Figure 6


%CD45RB^low of CD4^+KJ1-26^+
60
50
40
30
20
10
0
P
O
O+ E
O+ G
E
% CD4^+KJ1-26^+of CD4^+
0.8
0.6
0.4
0.2
0
SPLEEN
CLN

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Protection against Recurrent Ocular Herpes Simplex Virus Type 1 Disease after Therapeutic Vaccination of Latently Infected Mice

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The potential of therapeutic vaccination of animals latently infected with herpes simplex virus type 1 (HSV-1) to enhance protective immunity to the virus and thereby reduce the incidence and severity of recurrent ocular disease was assessed in a mouse model. Mice latently infected with HSV-1 were vaccinated intranasally with a mixture of HSV-1 glycoproteins and recombinant *Escherichia coli* heat-labile enterotoxin B subunit (rEtxB) as an adjuvant. The systemic immune response induced was characterized by high levels of virus-specific immunoglobulin G1 (IgG1) in serum and very low levels of IgG2a. Mucosal immunity was demonstrated by high levels of IgA in eye and vaginal secretions. Proliferating T cells from lymph nodes of vaccinated animals produced higher levels of interleukin-10 (IL-10) than were produced by such cells from mock-vaccinated animals. This profile suggests that vaccination of latently infected mice modulates the Th1-dominated proinflammatory response usually induced upon infection. After reactivation of latent virus by UV irradiation, vaccinated mice showed reduced viral shedding in tears as well as a reduction in the incidence of recurrent herpetic corneal epithelial disease and stromal disease compared with mock-vaccinated mice. Moreover, vaccinated mice developing recurrent ocular disease showed less severe signs and a quicker recovery rate. Spread of virus to other areas close to the eye, such as the eyelid, was also significantly reduced. Encephalitis occurred in a small percentage (11%) of mock-vaccinated mice, but vaccinated animals were completely protected from such disease. The possible immune mechanisms involved in protection against recurrent ocular herpetic disease in therapeutically vaccinated animals are discussed.

Ocular herpes simplex virus type 1 (HSV-1) infection is the major cause of nontraumatic blindness in developed countries. Initial infection occurs at the corneal epithelium, where, following replication, the virus enters the sensory nerve endings, travels along axons, and becomes latent in the trigeminal ganglion (TG) (14). The virus remains as a lifelong infection in the TG, probably undetected by the immune system. Under certain conditions, which include stress or exposure to UV light, the virus may reactivate, travel back down the nerve, and cause recurrent infection, most often in the cornea (20). The immune mechanisms involved in protection against HSV-1 infections include the recruitment of proinflammatory immune cells. In the case of the eye, these cells may lead to immunopathological disease by infiltrating the stroma, causing opacity and edema of this tissue. In certain cases, the cornea may become highly vascularized and thickened, particularly after repeated recurrent infections, resulting in severe stromal keratitis and visual impairment (29). Current methods of therapy involve the administration of antiviral drugs and corticosteroids, but these are not always effective and may in some cases exacerbate disease (13). Vaccination to prevent primary infection is problematic, since the virus is often acquired very early in life. Therefore, the development of a therapeutic vaccine for individuals with an established latent infection to prevent recurrent ocular disease or significantly decrease its severity is an attractive approach.

While a number of potential vaccine candidates have been shown to provide protection against primary ocular challenge, the efficacy of the few that have been tested in recurrent models of disease has been disappointing. In one study, a virion host shutoff mutant was tested as a live therapeutic vaccine against recurrent infection in the mouse. Although this live vaccine reduced the incidence of virus shedding following reactivation, the incidence of clinical ocular disease was unaffected (34). The use of subunit vaccines incorporating glycoprotein D in mice (16) and rabbits (21) has been similarly disappointing. These difficulties reflect the complex nature of the immune response in HSV-1 infection and the requirement for vaccination to modulate the protective components of immunity while at the same time limiting immunopathology. In this regard, immunohistochemical studies indicate that the initial response to recurrent infection in the eye involves an influx of neutrophils and macrophages together with $CD4^+$ and $CD8^+$ T cells, indicative of a proinflammatory Th1-type response. While this response is involved in viral clearance, it is also likely to drive the pathological damage to the eye that is associated with herpetic keratitis. At later times, the presence of B cells and anti-inflammatory cytokines (interleukin-10 [IL-10]) corresponds with the resolution of ocular disease (23, 27, 28). A successful therapeutic vaccine for ocular HSV-1 disease may, therefore, be one that can modulate the nature of the immune response, providing a higher degree of protection at the mucosal surface of the eye itself while limiting the proinflammatory effects of the virally induced Th1 response.

We have previously shown that intranasal immunization

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with a mixture of HSV surface glycoproteins in the presence of the recombinant *Escherichia coli* heat-labile enterotoxin B subunit (rEtxB) as adjuvant provided protection against HSV-1 infection. Following primary ocular challenge, immunized mice showed much reduced corneal disease and only limited spread of virus in the nervous system. The latter was evidenced by a reduction in zosteriform lesions and the incidence of latency in regions of the TG not served by the ophthalmic nerve. Immunized mice were also completely protected against the development of encephalitis, even under challenge conditions, in which the mortality in control, mock-vaccinated animals was as high as 95% (22). The immune response induced by our intranasal vaccine was characterized by the presence of strong secretory IgA responses to HSV-1 antigens in mucosal washings and serum neutralizing antibodies. The dominance of IgG1 in the serum antibody response together with the presence of high levels of IL-4 and IL-10 in lymph node cell cultures from immunized mice suggested that the anti-HSV-1 response was Th2 dominated. However, there was also some gamma interferon (IFN-γ) production in such cultures, indicating that Th1 immunity was also present. We hypothesized that the type of immune response generated to our vaccine may be compatible with modulating immunity in the latently infected animal. In order to test this hypothesis, we have utilized a well-characterized mouse model of recurrent herpetic eye disease (24, 25). Mice infected by corneal scarification in the presence of passive antibodies develop mild epithelial disease in 80 to 100% of cases. In our hands, this leads to the establishment of latency in approximately 93% of animals, as determined by inoculation of medium from 5-day TG explant cultures onto Vero cells (26). A slightly modified version of this model used by another laboratory shows that 80 to 100% of mice had latent virus in the TG following ocular infection (16). Reactivation of virus by exposure to UV light occurs in approximately 60% of mice, as determined by viral shedding, incidence of disease, and cell infiltration (24, 28). We describe here the effects of vaccination on the development of recurrent herpetic ocular disease in this mouse model.

MATERIALS AND METHODS

Reactivation model. Female, specific-pathogen-free NIH mice obtained from Harlan Olac, Bicester, United Kingdom, were maintained in the School of Medical Sciences, University of Bristol, Bristol, United Kingdom. At 8 weeks of age, mice were inoculated with HSV in order to establish latent infection in the TG, according to the method described previously (24). Briefly, mice were inoculated intraperitoneally with human serum (Harlan Sera-Lab, Ltd., Loughborough, United Kingdom), containing HSV-1 serum neutralizing antibodies, which was assayed to determine the serum antibody titer required to give 50% virus plaque reduction (50% effective dose [ED_{50}]) (25). Serum was diluted in phosphate-buffered saline (PBS) to give an ED_{50} of 8,000 (24). After 24 h, mice were anesthetized (100 mg of ketamine per kg of body weight [Parke-Davis, Pontypool, United Kingdom] mixed with 10 mg of xylazine per kg [Bayer, Bury St. Edmunds, United Kingdom]) and infected with 10^6 PFU of HSV-1 McKrae in a 5-μl drop of medium by ocular scarification of the right cornea with a 26-gauge needle (24). Six weeks after infection, the right eyes of all mice were checked for the presence of any abnormalities, and such mice were discarded. The remaining mice were immunized intranasally three times at 10-day intervals with either 10 μg of HSV-1 glycoproteins, prepared from Vero cells infected with live HSV-1, or mock glycoproteins, prepared from uninfected Vero cells, each mixed with 20 μg of rEtxB to give a final volume of 47 μl (22). Two to 4 weeks after the final immunization, animals were anesthetized and placed with their right eye proptosed below a Hanovia lamp (emitting a peak of 4.02 mJ/cm^2 s at 320 nm), and the right corneas and lids were irradiated for 90 s in order to induce reactivation (25).

Measurement of antibody responses. Individual mice were bled from the tail vein 4 weeks after corneal scarification and 1 week after the final immunization, and the serum was stored at −20°C. Serum from mice infected without passive immunization was collected for use as a positive control. Eye and vaginal washings were collected from mice anesthetized with halothane by pipetting 20 μl of PBS up and down on the surface of each eye 10 times or with 50 μl of PBS pipetted in and out of the vagina 20 times. Samples collected from individual mice over several days were pooled and stored at −20°C.

Sera were analyzed for the presence of HSV-1-specific antibodies, as described previously (7). Briefly, assay plates coated with rabbit anti-HSV-1 (Dako, Ltd., High Wycombe, United Kingdom) were incubated with 1% bovine serum albumin in PBS, followed by HSV-1 antigen, mouse serum, and a rabbit anti-mouse Ig-horseradish peroxidase (HRP) conjugate (Dako Ltd.), with *O*-phenylenediamine (Sigma) as a substrate.

In order to measure the levels of virus-specific IgA in mucosal fluids, the conjugated secondary antibody was replaced with an HRP-conjugated goat anti-mouse IgA (Sigma). The level of IgG1 and IgG2a in the sera was measured with HRP-conjugated rat anti-mouse IgG1 or IgG2a, respectively. The endpoint titers for individual samples were determined by linear regression analysis.

The presence of neutralizing antibodies in sera of infected mice and mice immunized with HSV-1 or mock glycoproteins was determined by pooling aliquots of serum from individual mice in each group, collected as described above, for use in a plaque reduction assay. The ED_{50} was calculated by weighted probit analysis (2), and the titers are given as reciprocals.

Assessment of T-cell responses. Single-cell suspensions of lymphocytes in Hanks' balanced saline solution (HBSS) (Gibco, Paisley, United Kingdom) containing 20 mM HEPES buffer (GIBCO) were prepared from draining lymph nodes, removed from mice either 4 weeks after final immunization or UV irradiation by agitation through wire mesh with a glass rod. Lymphocytes were washed and then cultured at 10^6 cells per ml in minimal essential medium α (α-MEM), supplemented with 20 mM HEPES, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 4 mM L-glutamine (Gibco), 50 μM 2-mercaptoethanol (Sigma), and 0.5% normal autologous mouse serum in 25-cm^2 flasks. Cells were cultured in the presence of UV-inactivated virus (prepared from serum-free supernatant of infected Vero cells) at a predetermined, optimal concentration of 1.5×10^5 PFU/ml, an equivalent dilution of mock virus for assessment of nonviral responses, or medium alone (data not shown). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. Aliquots of 100 μl were removed on desired days after initiation of the cultures and placed in triplicate into wells of a 96-well plate for assessment of [^{3}H]thymidine incorporation by standard assay techniques (12).

Additional aliquots of cells were removed for assessment of cytokine levels by a previously described method (3). Briefly, cell samples, set up in triplicate, were cultured overnight at 37°C in a humidified atmosphere of 5% CO_2 in capture antibody-coated (rat anti-mouse cytokines) enzyme-linked immunosorbent assay (ELISA) plates, before detection with biotinylated rat anti-mouse cytokines (BD Pharmingen, San Diego, Calif.). Thus, cytokine production by cells over a defined period of culture could be assessed under conditions in which the effect of cytokine lability was minimized. Cytokine levels were calculated by regression analysis against standard curves produced with the appropriate recombinant cytokine (BD Pharmingen, San Diego, Calif.).

Analysis of ocular disease and isolation of virus from eye washings following reactivation of latent virus. Following corneal scarification, eyes were examined on days 1, 3, and 7 with a Zeiss 105L slit lamp microscope (Zeiss, Welwyn Garden City, United Kingdom) for development of epithelial ulcers, stromal disease, and uveitis. The eyes were also checked prior to immunization, and any mice with damaged corneas were removed from the experiment. Similarly, mice were analyzed following UV irradiation on days 1, 2, 4, 6, 10, and 14 for ocular disease as well as for spread of virus to other areas, resulting in ulceration and edema of the eyelid as well as zosteriform herpetic lesions in the skin at sites served by the TG (the snout and lower jaw). The severity of each disease parameter was also scored in those mice with disease (1 = mild disease and 5 = most severe disease). Piloerection, loss of weight, hunched posture, and a significant defect in righting reflex were taken as signs of encephalitis, and such animals were killed by cervical dislocation.

Eye washings were collected on day 0 to check for any spontaneous reactivation, as well as on days 2, 3, 4, 5, and 6, from irradiated eyes by pipetting 20 μl of culture medium onto the surface of the proptosed eye 10 times and transferring the washes to Vero cells for isolation of virus (30).

Statistical analysis. Significant differences in the immune response and clinical disease score between groups of mice were determined by Student's *t* test. Incidence data were analyzed with the χ^2 test.

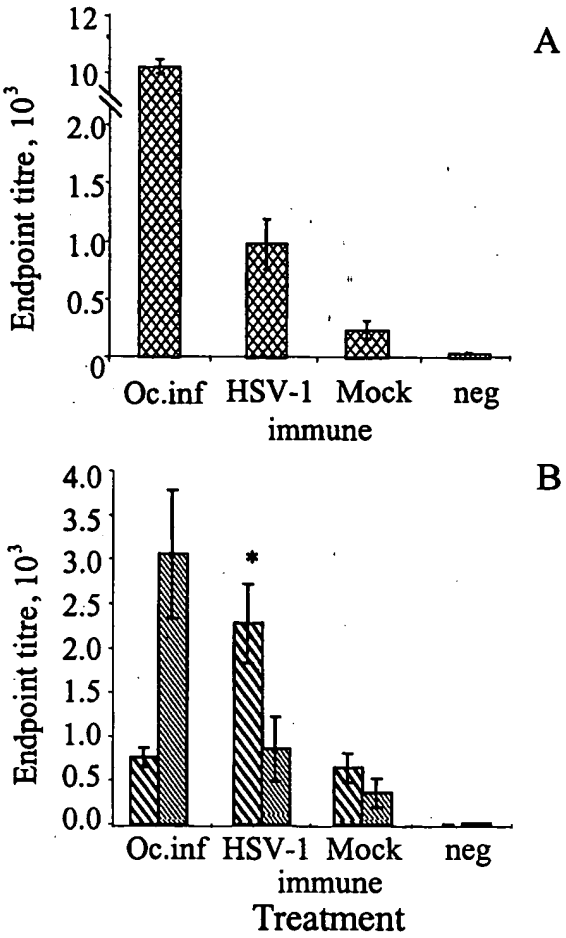

FIG. 1. HSV-1-specific serum responses in mice 4 weeks after corneal scarification with HSV-1 McKrae and 1 week following HSV-1 immunization and mock immunization of latently infected mice as determined by ELISA. The serum Ig responses to HSV-1 antigens were analyzed by regression analysis, and the endpoint titers were determined (A). Endpoint titers were also determined for HSV-1-specific serum IgG1 (bold hatched bars) and IgG2a (light hatched bars) in IgG subclass-specific ELISA (B). Mean values with standard error of the mean were calculated from groups of 20 immunized mice and 5 mice following ocular infection (Oc.inf). Sera from naïve mice were included as the negative control (neg). An asterisk shows a significant difference in response between HSV-1-vaccinated and mock-vaccinated mice as judged by Student's t test ($P < 0.01$). The data presented here are representative of three similar experiments.

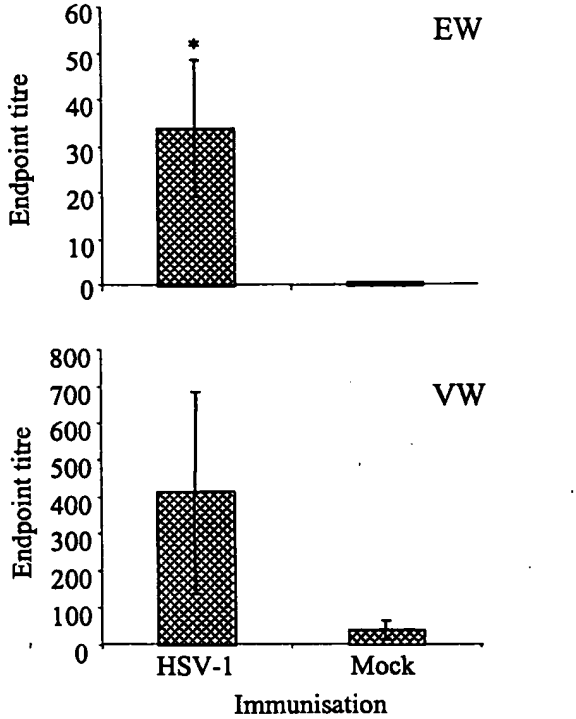

FIG. 2. HSV-1-specific IgA in mucosal washings collected from the eye (EW) and vagina (VW) of HSV-1-vaccinated and mock-vaccinated mice as determined by ELISA. Endpoint titers were calculated by regression analysis for individual mice ($n = 20$), and mean values ± standard errors were determined. An asterisk shows significant difference between HSV-1-vaccinated and mock-vaccinated mice as judged by Student's t test ($P < 0.05$).

RESULTS

Modulation of the anti-HSV-1 antibody response in latently infected mice. High levels of anti-HSV-1 antibodies were detected in the serum of mice that had been infected with HSV-1 by corneal scarification, with endpoint titers in excess of 1:10,000 (Fig. 1A). Serum from passively immunized mice infected by corneal scarification had low levels of anti-HSV-1 antibodies following immunization with mock glycoproteins (prepared from uninfected Vero cells) mixed with rEtxB as adjuvant. In contrast, immunization with glycoproteins from HSV-1-infected Vero cells mixed with rEtxB dramatically enhanced the levels of anti-HSV-1 antibodies in latently infected animals. The mean endpoint titer in mock-immunized mice was 1:236, compared to 1:971 for the immunized group ($P < 0.01$).

Analysis of the serum IgG subclasses following corneal scarification alone showed that IgG2a was the dominant subclass, resulting in an IgG1/IgG2a ratio of 0.25 (Fig. 1B). In contrast, following HSV-1 immunization of latently infected mice, IgG1 was the dominant subclass, with an endpoint titer of 1:2,276 compared with IgG2a at 1:858. The ratio of IgG1 to IgG2a following immunization in the presence of rEtxB was 2.65. This is a considerable enhancement of the IgG1 and IgG2a responses observed in mock-immunized mice with an IgG1 endpoint titer of 1:640 compared with 1:360 for IgG2a, giving an IgG1/IgG2a ratio of 1.78.

Further characterization of HSV-1-specific antibody responses was undertaken, using pooled serum samples, to assess virus neutralization. Serum from infected only mice gave virus neutralization titers of 1:1,083. Latently infected mice immunized with HSV-1 glycoproteins gave an ED_{50} titer of 1:107, which was in marked contrast to mock-immunized mice, which gave an ED_{50} titer of 1:7 (data not shown).

In addition to the stimulation of serum antibodies to HSV-1, immunization of latently infected mice triggered the production of high levels of secretory IgA, particularly at the ocular surface, local to the site of administration, as well as the vagina, a more distant site (Fig. 2). The mean endpoint titer for eye washings from immunized mice, taken 1 week to 10 days following the final immunization, was 1:34. This is a significant

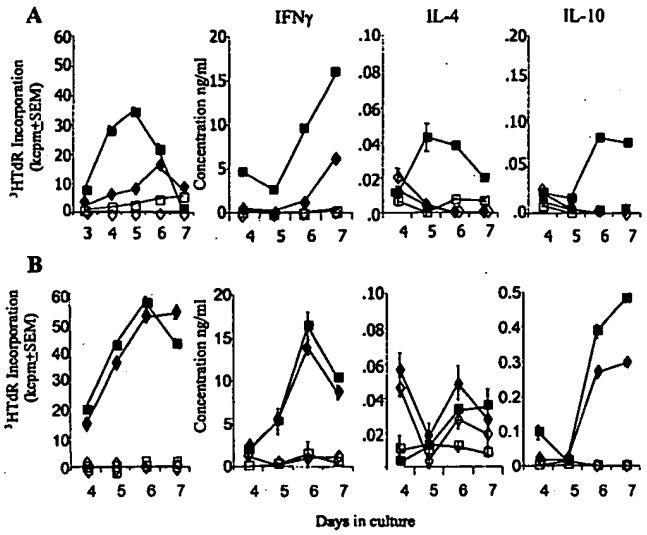

FIG. 3. Draining lymph node cells from HSV-1-vaccinated (squares) and mock-vaccinated (diamonds) mice prior to reactivation (A) or postreactivation (B), cultured in vitro with HSV-1 (solid symbols) or mock antigen (open symbols), were analyzed for proliferative responses by [^{3}H]thymidine (^{3}HTdR) incorporation and secretion of cytokines IFN-γ, IL-4, and IL-10. Mean values ± standard errors of triplicate cultures are shown. The data presented here are representative of three similar experiments.

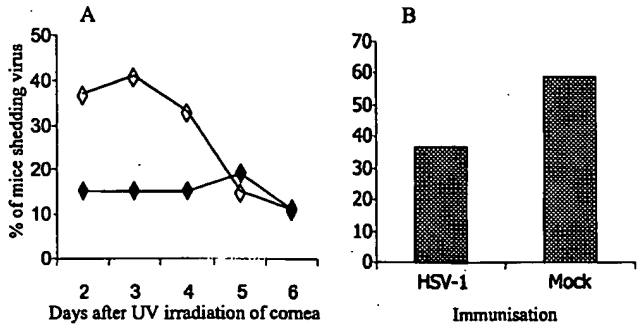

FIG. 4. Percentage of HSV-1-vaccinated (solid symbols) and mock-vaccinated (open symbols) mice shedding virus in eye washings days 2 to 6 after UV-induced reactivation of latent virus (A) and total percentage of mice shedding virus on at least 1 day (B). A significant reduction in viral shedding was observed in HSV-1-vaccinated mice (χ^2 test, $P < 0.01$).

increase compared with that of the mock-immunized controls, which gave a mean endpoint titer of 1:0.5 ($P < 0.05$). The presence of IgA in vaginal washings of immunized mice resulted in a mean endpoint titer of 1:412 compared with 1:40 for the mock-immunized mice. This difference, however, was not significant due to the large variation between individuals within the same group.

Effects of immunization on anti-HSV-1 T-cell responses in latently infected animals. The effect of immunization of latently infected mice on T-cell responses was investigated with lymphocytes isolated from draining lymph nodes either 4 weeks after immunization and prior to reactivation or 4 weeks after subsequent UV-induced reactivation of virus. Cells were cultured in the presence of UV-inactivated HSV-1 or mock antigen, and T-cell proliferation and cytokine production were assessed on days 4 to 7. The results showed strong T-cell proliferative responses to HSV-1 antigens by cells from latently infected, HSV-1-immunized animals (Fig. 3A). A higher and more rapid response indicative of a secondary recall response was observed with cells following reactivation of latent virus in both HSV-1- and mock-immunized animals, with peak incorporation of [^{3}H]thymidine in excess of 50 kcpm on days 6 to 7 (Fig. 3B). The response of these cell cultures to mock antigen was negligible, indicating that proliferation of T cells was specific for viral antigens.

Analysis of the cytokines secreted by proliferating T-cell cultures indicated that following immunization, IFN-γ was the main cytokine secreted, with low levels of IL-10 and IL-4 compared to the results for cells from mock-immunized mice and those cultured with mock antigen. Following reactivation, a more rapid IFN-γ response was seen from cells from both HSV-1-immunized and mock-immunized mice in response to HSV-1 antigen, with peak concentrations of IFN-γ observed on day 6 of 16 and 14 ng/ml, respectively. Interestingly, the presence of IL-10 was observed in cells cultured following reactivation of virus, but was significantly enhanced in cell cultures from HSV-1-immunized mice compared to mock-immunized mice, with levels of 0.48 ng/ml on day 7 compared with 0.29 ng/ml, respectively. Increased production of IL-10 in cultures from immunized mice following reactivation was observed in each of three similar experiments. While some IL-4 was detected, the levels observed were very low, making the differences between cultures difficult to distinguish.

Clinical disease and viral shedding following reactivation of latent virus. Analysis of the eyes of passively immunized mice infected by corneal scarification revealed that 97.5% of mice developed corneal ulcers by day 3. Of these, 50.5% developed some signs of stromal disease and 25% demonstrated uveitis, although none of these developed severe disease symptoms and thus had recovered by day 7 following infection.

The effect of modulating the immune response to HSV-1 antigens on recurrent herpetic ocular disease was determined following reactivation of latent virus by exposure to UV light. Eye washings were collected immediately prior to UV irradiation to ensure that the presence of virus was not the result of spontaneous shedding; such shedding is rare in this model (21, 22). Indeed, no spontaneous viral shedding was detected in these experiments, indicating that any virus present was most likely due to UV-induced reactivation. The main period for detection of reactivated virus in eye washings taken from mock-immunized mice was represented by days 2, 3, and 4, with a peak incidence on day 3 of 41% (Fig. 4A). In contrast, reactivated virus was only observed in 15% of HSV-1-immunized mice on days 2 to 4, with a peak incidence of 19% occurring on day 5. A significant reduction in the overall incidence of mice shedding virus, over the 10 days following reactivation, was observed between HSV-1-immunized (37%) and mock-immunized (59%) mice (Fig. 4B; χ^2 test, $P < 0.01$).

Reactivation of HSV-1 is partly a consequence of corneal damage caused by exposure to UV light resulting in the development of some corneal abnormalities immediately following UV treatment in both groups of mice. Such abnormalities, however, are short term, and only those mice with HSV-1-associated disease continue to show signs of corneal disease

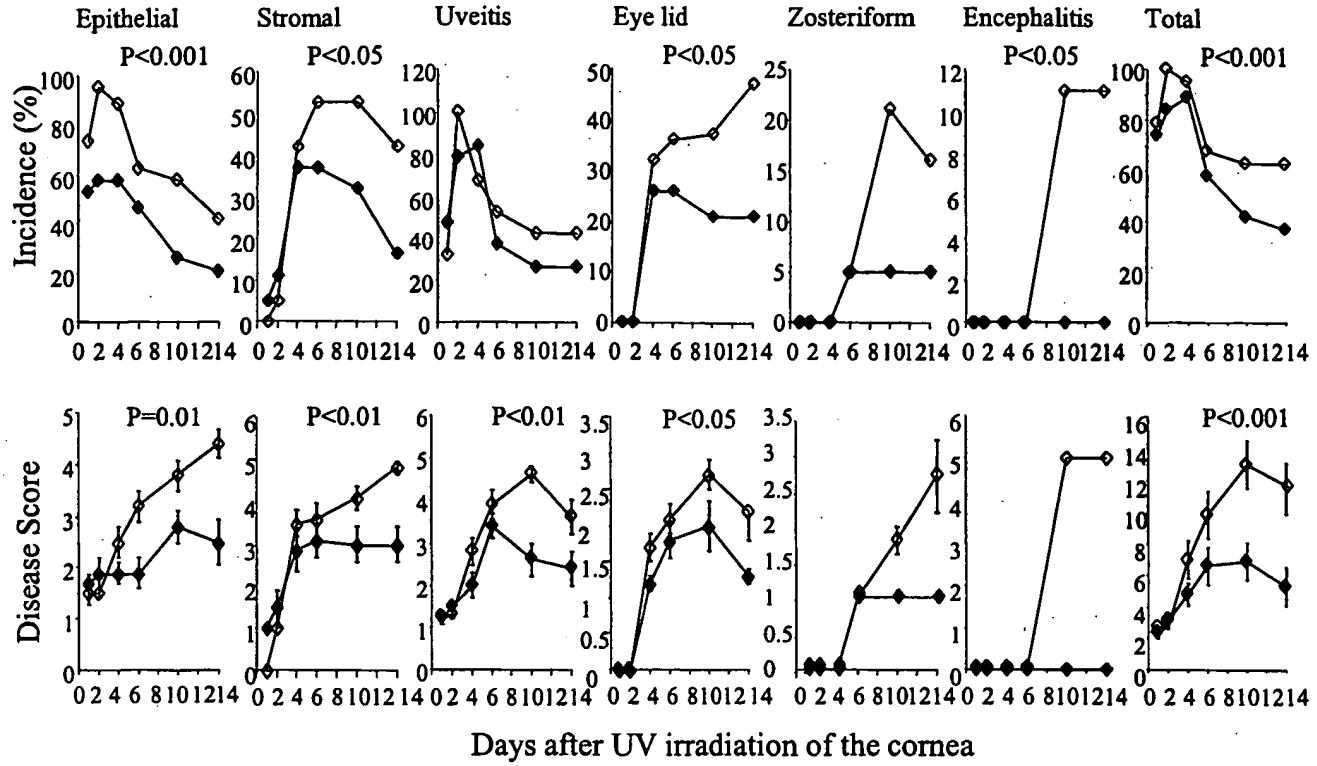

FIG. 5. Incidence and severity of the different clinical parameters following reactivation of latent virus in HSV-1-vaccinated (solid symbols) and mock-vaccinated (open symbols) mice. Disease scores run from 0 (no disease) to 5 (very severe disease). Each group started with 20 mice. Disease scores represent mean values of mice with disease in each group ± standard errors. Significant differences between HSV-1- and mock-vaccinated mice were determined by Student's *t* test.

and increased severity. Thus, the incidence of epithelial disease in HSV-1-vaccinated mice was significantly reduced over the 14 days analyzed compared with that in the mock-vaccinated mice (χ^2 test, $P < 0.001$), with a maximum of 58% compared with 95%, respectively, on day 2 (Fig. 5). Stromal disease levels were initially similar in both groups of mice, but those vaccinated with HSV-1 peaked at 37% on days 4 to 6 and then declined steadily so that only 16% were affected by day 14. Such disease in mock-immunized mice increased significantly to a peak of 53% by day 6, staying at this level until day 10, before declining to 42% by day 14 (χ^2 test, $P < 0.05$%). No significant difference in the incidence of uveitis between the two groups was observed, with 84% of HSV-1-immunized mice showing signs of uveitis on day 4 compared with 100% for mock-immunized animals on day 2. Lid disease was first observed in both groups on day 4, with 26% of HSV-1-immunized mice affected compared with 32% of mock-immunized mice. The incidence of lid disease in mock-immunized mice continued to increase significantly to a peak of 47% by day 14. In contrast, a significantly lower incidence of lid disease was observed up to day 14 in HSV-1-immunized mice (χ^2 test, $P < 0.05$), with only 21% affected by day 10. Likewise, zosteriform spread was observed in 5% of mice from both groups on day 6, but no further development of this form of the disease was observed in HSV-1-immunized mice. In contrast, in the mock-immunized mice, the incidence of zosteriform spread increased further to 21%. By day 10, 11% of mock-immunized mice developed encephalitis and were killed by cervical dislocation. Taken together, a significant reduction in the incidence of HSV-1-associated disease was observed in HSV-1-immunized versus mock-immunized mice over the days analyzed following reactivation of virus by UV irradiation (χ^2 test, $P < 0.001$).

In addition to this clear difference in disease incidence, analysis of disease severity in the mice with clinical symptoms further highlighted the protective efficacy of intranasal vaccination of latently infected animals. Although similar levels of ocular disease were observed in both groups of mice in the first few days following reactivation of virus, the mice vaccinated with HSV-1 glycoproteins and rEtxB recovered quickly from UV damage, whereas mock-vaccinated animals continued to develop disease and disease severity increased. In particular, a significant reduction in disease severity was observed for epithelial disease (Student's *t* test, $P < 0.01$), stromal disease (Student's *t* test, $P < 0.01$), uveitis (Student's *t* test, $P < 0.01$), and lid disease (Student's *t* test, $P < 0.05$). Furthermore, HSV-1-vaccinated mice developed less severe zosteriform lesions, and none showed signs of encephalitis. Thus, there was a significant difference in total incidence and severity of recurrent clinical disease between the two groups (Fig. 5; Student's *t* test, $P < 0.001$).

DISCUSSION

In this paper, we show that intranasal vaccination of latently infected mice with a mixture of HSV-1 glycoproteins and EtxB

can stimulate protective immunity in a well-characterized mouse model of recurrent ocular herpetic disease. The immune response generated in this way was characterized by increased anti-HSV-1 antibody levels of local IgA and serum IgG, an increased IgG1/IgG2a ratio, and elevated IL-10 production by lymphocytes from local lymph nodes. Vaccination was also associated with a reduction in the percentage of mice shedding virus at the corneal surface, together with reduced incidence of disease induced by UV irradiation. Furthermore, the mice that developed clinical symptoms showed enhanced recovery and limited spread of virus through the nervous system, with only a few vaccinated mice developing lid disease and zosteriform lesions. None showed signs of encephalitis.

Corneal infection in this model results in approximately 90% of mice becoming latently infected with HSV-1 in the ophthalmic region of the TG (16, 26). The presence of latent virus was also shown to correlate well with the development of ocular disease following infection; thus, in this set of experiments, which showed 97.5% of mice developing some signs of ocular disease, the presence of latent virus would be comparable with results of 80 to 100% from previous studies.

UV irradiation itself damages the corneal epithelium and causes uveitis, events that are thought to be necessary for reactivation of virus in the TG (28). Such damage, however, is short lived, so that only those mice with viral reactivation develop the more severe stromal disease associated with herpes stromal keratitis (HSK). Consequently, in this study, a high percentage of both the immunized and mock-immunized mice developed corneal disease following UV irradiation. However, by day 6, most mice had recovered from UV-induced damage, resulting in a fall in the incidence of corneal disease. This was particularly evident in the case of uveitis, from which only 38% of HSV-1-vaccinated mice were affected following initial damage compared with 55% of mock-vaccinated animals, which had uveitis that continued to increase in severity. Similarly, for both epithelial and stromal disease, the severity of disease in the mock-immunized mice with clinical symptoms continued to increase for the duration of the experiment, while HSV-1-vaccinated mice developed less severe disease. Spread of virus from the initial site of infection to other areas within the same dermatome led to lid disease in 48% of mock-immunized mice, whereas only 25% of HSV-1-immunized mice developed the disease, with reduced severity. The further spread of virus causing zosteriform lesions was only observed in 5% of HSV-1-immunized mice, and the severity of these lesions was also low. In addition, none of the HSV-1-immunized mice developed signs of encephalitis. These results indicate that HSV-1 vaccination results in a reduction in the spread of virus through the nervous system when compared with mock-vaccinated mice. Overall, there was a significant reduction in both the incidence and severity of clinical disease in vaccinated mice following reactivation of latent virus, compared with the level in mock-vaccinated animals. Furthermore, in this model, recurrent herpetic eye disease is usually associated with shedding of virus in the tears (25). Therefore, isolation of virus from eye washings provided an indicator of the presence of reactivating virus. In previous studies, approximately 60% of mice shed virus in the tears following UV irradiation of the cornea (17, 24, 25, 34), which is comparable with the incidence in the mock-immunized mice in the present experiments. In contrast, this incidence was only 37% in HSV-1-immunized mice. A similar reduction in viral shedding was also observed in mice vaccinated with the virion host shutoff (*vhs*)-defective mutant of HSV-1 (17). At present, it is not clear whether this lower incidence is due to antibody-mediated neutralization of virus at the surface of the eye or a reduction in the number of reactivating events in the TG.

In the present study, the immune response induced by vaccination of latently infected mice was similar to that obtained by vaccination of naïve mice (22). High levels of virus-specific serum Ig, comparable to that obtained following corneal scarification of mice with live HSV-1, were detected by enzyme-linked immunosorbent assay (ELISA) and by neutralization in plaque reduction assays. Of particular importance for protective immunity at the corneal surface was the induction of virus-specific IgA in eye washings, the site of infection and recurrent disease. While some mice also developed high levels of IgA at the more distant mucosal site, the vagina, this was highly variable, which may be a consequence of the transient nature of IgA in mucosal washings. However, the presence of virus-specific IgA at the ocular surface, the site of infection and recurrence, has been shown to play a part in protection against ocular disease and corneal scarring (9). Analysis of the spread of virus in in vitro cultures has shown that virus-specific antibodies prevent spread between epidermal cells by neutralization during transmission across the intercellular gap (19). Similar mechanisms may also limit the spread of virus between epidermal cells and axonal termini in vivo (11). Thus, following reactivation, virus-specific antibody is likely to play an important role in limiting viral shedding from the nerve endings in the cornea and the spread of virus between neighboring corneal cells once they are infected.

Other mechanisms that may be involved in reduced corneal disease include the switch from the Th1-dominated, proinflammatory immune response observed in mice after a primary infection to a more balanced Th1/Th2-type response associated with anti-inflammatory cytokines observed following immunization. This approach contrasts with that aimed at providing protection against vaginal challenge with the closely related HSV-2. In these studies, induction of strong Th1-type responses enhanced viral clearance, resulting in reduced incidence and severity of genital lesions (10). However, following infection of the eye, the persistence of proinflammatory cytokines following viral clearance results in continued influx of Th1 immune cells, leading to opacity and vascularization of this highly specialized, normally transparent organ (6). Thus, the potential to modify the immune response to reactivating virus by immunization with rEtxB toward a more balanced Th1/Th2-type anti-inflammatory response, as observed in previous studies after immunization of naïve mice (22), was considered to be advantageous. It is also possible, however, that the major factor that affects the protection from disease observed in the present studies is the increase in levels of IL-10. This may reflect the activation of a T regulatory population following immunization that is able to suppress the immunopathological processes associated with ocular disease.

Accordingly, a switch in the dominance of IgG1 and IgG2a was observed, with the change in the IgG1/IgG2a ratio from 0.25 following ocular infection to 2.65 following HSV-1 immunization of latently infected animals indicating the potency of

rEtxB to modulate the immune response. Interestingly, serum from mock-immunized mice also showed higher levels of IgG1 than IgG2a, resulting in a ratio of 1.78, suggesting that the use of rEtxB alone is capable of influencing the balance of the IgG subclasses. This is consistent with our observations that show rEtxB can, when given alone, modulate Th1 responses to autoantigens mediating protection in animal models of autoimmune disease (18). Several studies have also shown the importance of the anti-inflammatory cytokines IL-4 and IL-10 in the resolution of stromal keratitis (4, 5). The involvement of IL-4 in the resolution of corneal disease, however, is contradictory, the presence of IL-4 also being associated with development of HSK (1) and increased mortality (15). Recent studies have indicated that the mode of action of IL-4 is dependent on the other cell types present, such as $CD4^+$ or $CD8^+$ T cells (8). While we have not yet assessed the effects of immunization on the activation of HSV-specific cytotoxic T lymphocytes, the clear association with increased IL-4 and IL-10 levels suggests that such responses are unlikely to be a key feature of vaccination in this way. Further investigations will be required to determine this. Different roles for IL-4 and IL-10 have also been demonstrated following administration of either IL-4 or IL-10 DNA by different routes prior to ocular infection. The use of IL-4 DNA resulted in a significant switch toward a Th2 subset balance, whereas IL-10 did not (4). The involvement of IL-4 in this study is not clear; the level of IL-4 seen following immunization of these latently infected animals is comparable to that seen with previous studies involving immunization of naïve mice (22). Analysis of the cytokines following reactivation of virus showed similar levels of IL-4 in both HSV-1- and mock-immunized mice. Whereas, although only low levels of IL-10 were observed following immunization, this was greatly enhanced upon reactivation, in excess of that seen in the mock-immunized controls. The induction of IL-10 has been shown to be of primary importance in regulation of inflammatory responses and the resolution of HSK (1, 4, 5). The presence of IL-10 following HSV-1 corneal infection has been reported to be associated with suppression of certain chemokines, such as macrophage inflammatory protein 2 (MIP-2) and MIP-1α, resulting in a reduction in the migration of inflammatory cells to the cornea (31, 35). In addition, IL-10 has been shown to down-regulate expression of proinflammatory cytokines such as IL-2 and IL-6 (32). Further studies are required to determine the mechanism involved in IL-10 secretion. The observation that only local administration of IL-10 was effective led to the conclusion that either corneal epithelial cells or infiltrating immune cells may be able to secrete IL-10 (35). However, the cytokine profile observed here was similar to that following exposure of human monocytes to rEtxB, with high IFN-γ and IL-10 levels (33). This cytokine profile was consistent with that described for a subset of IL-10-secreting lymphocytes that possess regulatory properties (Tr1 cells) and that have been shown to be capable of preventing or treating Th1-mediated autoimmune diseases. This suggests that the ability of EtxB to trigger activation of T-regulatory cells (18) may be an important feature in modulating the immune-mediated damage associated with herpetic ocular disease.

In conclusion, we have demonstrated the potential of therapeutic vaccination to protect against recurrent ocular HSV-1 infection. Mucosal administration of viral antigens, together with the use of the potent immunomodulator rEtxB, induced an immune response that protected against corneal damage and blindness. The possible mechanisms involved include a rapid IgA antibody response at the corneal surface, enabling neutralization of virus. The presence of high levels of the IgG1 subclass of HSV-1-specific antibody helped give a more balanced Th1/Th2 and anti-inflammatory response. The induction of IL-10 is involved in the regulation of inflammatory responses, possibly due to the induction of Tr1 cells by rEtxB. Taken together, the modulation of both humoral and cell-mediated immune responses resulted in a significant reduction in the incidence and severity of this immunopathological disease.

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